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EIF2 α phosphorylation is regulated in intracellular amastigotes for generation of infective *Trypanosoma cruzi* trypomastigote forms

Fabricio Castro Machado^{1*}, Paula Bittencourt-Cunha^{1*}, Amaranta Muniz Malvezzi^{1*}, Mirella Árico¹, Santiago Radio^{2,3}, Pablo Smircich^{2,3}, Martin Zoltner⁴, Mark C. Field^{5,6}, Sergio Schenkman¹

¹Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil

²Department of Genomics, Instituto de Investigaciones Biológicas Clemente Estable, Ministerio de Educación y Cultura, Montevideo, Uruguay.

³Laboratory of Molecular Interactions, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay.

⁴Drug Discovery and Evaluation, Center for Research of Pathogenicity and Virulence of Parasites, Charles University in Prague, BIOCEV, Průmyslová 595252 50 Vestec, Czech Republic

⁵Division of Biological Chemistry and Drug Discovery, University of Dundee, Dundee, DD1 5EH, UK

⁶Institute of Parasitology, Czech Academy of Sciences, 37005 Ceske Budejovice, Czech Republic

Correspondence

Sergio Schenkman, R. Pedro de Toledo 669 L6A, São Paulo SP, 04039-032, Brazil

Email: sschenkman@unifesp.br

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* These authors contributed equally to this work.

Running title

Trypanosoma cruzi eIF2 α phosphorylation role.

Abstract

Trypanosomatids regulate gene expression mainly at the post-transcriptional level through processing, exporting and stabilizing mRNA and control of translation. In most eukaryotes, protein synthesis is regulated by phosphorylation of eukaryotic initiation factor 2 (eIF2) at serine 51. Phosphorylation halts overall translation by decreasing availability of initiator tRNA^{met} to form translating ribosomes. In trypanosomatids the N-terminus of eIF2 α is extended with threonine 169 the homologous phosphorylated residue. Here we evaluated whether eIF2 α phosphorylation varies during the *Trypanosoma cruzi* life cycle, the etiological agent of Chagas' disease. Total levels of eIF2 α are diminished in infective and non-replicative trypomastigotes compared to proliferative forms from the intestine of the insect vector or amastigotes from mammalian cells, consistent with decreased protein synthesis reported in infective forms. eIF2 α phosphorylation increases in proliferative intracellular forms prior to differentiation into trypomastigotes. Parasites overexpressing eIF2 α ^{T169A} or with an endogenous CRISPR/Cas9-generated eIF2 α ^{T169A} mutation were created and analysis revealed alterations to the proteome, largely unrelated to the presence of μ ORF in epimastigotes. eIF2 α ^{T169A} mutant parasites produced fewer trypomastigotes with lower infectivity than wild type, with increased levels of sialylated mucins and oligomannose glycoproteins, and decreased galactofuranose epitopes and the surface protease GP63 on the cell surface. We conclude that eIF2 α expression and phosphorylation levels affect proteins relevant for intracellular progression of *T. cruzi*.

KEYWORDS

Trypanosoma cruzi, translation, differentiation, virulence, phosphorylation, eIF2

1. INTRODUCTION

Changes to temperature, pH, oxidative state, nutrient and ionic concentrations are all environmental challenges that *Trypanosoma cruzi* parasites encounter during their life cycle (Souza, 2009). Consequently, the parasite changes gene expression, metabolism and morphology to adapt to these conditions (Piacenza *et al.*, 2009; Goldenberg *et al.*, 2011; Moretti *et al.*, 2013; Amorim *et al.*, 2017). How parasites sense environmental conditions, and which signals initiate alterations to gene expression remain poorly characterized. Few gene specific transcription promoters are present in trypanosomes with the exception of the spliced leader, ribosomal RNA promoters and expression site promoters in *Trypanosoma brucei* (Haile *et al.*, 2007; Cribb *et al.*, 2010; Martinez-Calvillo *et al.*, 2010). Transcription by RNA polymerase II produces long transcripts of non-related genes, which are processed by *trans*-splicing and polyadenylation to generate mature mRNAs prior to export to the cytoplasm (Palenchar *et al.*, 2006; Zinoviev *et al.*, 2012; Clayton, 2019). Furthermore gene expression continues to be regulated within the cytoplasm by selection of which mRNAs are stored, degraded or translated in each specific life stage (Lahav *et al.*, 2011; Kramer, 2012; Schwede *et al.*, 2012; Fritz *et al.*, 2015).

Differentiation of *T. cruzi* proliferative forms, either in the insect vector (epimastigotes) or inside mammalian cells (amastigotes), into non-proliferative infective trypomastigotes is accompanied by global decreases in transcription and translation (Elias *et al.*, 2001; Ferreira *et al.*, 2008; Smircich *et al.*, 2015). Recent reports attribute key roles for RNA-binding proteins (RBPs) in regulating mRNA availability for translation through storage and degradation processes in trypanosomes (Cassola, 2011; Das *et al.*, 2012; Guerra-Slomp *et al.*, 2012; Kolev *et al.*, 2012; Perez-Diaz *et al.*, 2012; Umaer *et al.*, 2015; Belew *et al.*, 2017; Mugo *et al.*, 2017). It is assumed that RBPs bind to specific mRNA regions to promote engagement with the translation machinery or the multiple classes of RNA granules (Markmiller *et al.*, 2018). Phosphorylation of eIF4 paralogs has been suggested as a regulatory mechanism governing targeting of distinct mRNA species (Pereira *et al.*, 2013), whilst translation

also correlates with phosphorylation levels of eIF2, probably allowing response to altered conditions during life stage transition (Kramer *et al.*, 2008; Chow *et al.*, 2011; Tonelli *et al.*, 2011; Cloutier *et al.*, 2012; Avila *et al.*, 2016).

eIF2 is a heterotrimer (α , β and γ) and in *T. cruzi*, the α -subunit is phosphorylated at threonine 169, which is homologous to a highly conserved serine at position 51 in most eukaryotes due to the presence of an N-terminal extension (Moraes *et al.*, 2007). eIF2 α phosphorylation inhibits GDP to GTP exchange promoted by the eIF2B guanine exchange factor (GEF), decreasing the availability of the translation complex eIF2^{GTP} and initiator methionine tRNA, tRNA^{Met} (Pavitt *et al.*, 1998; Krishnamoorthy *et al.*, 2001; Kashiwagi *et al.*, 2016; Bogorad *et al.*, 2017). Decreased levels of eIF2^{GTP}-tRNA^{Met} reduces global protein synthesis while allowing translation of mRNAs containing multiple initiation sites, or μ ORFs. These mRNAs encode transcription factors that regulate genes involved in remediating cellular stresses (Pavitt *et al.*, 1998; Hinnebusch, 2000; Sonenberg *et al.*, 2009; Palam *et al.*, 2011; Baird *et al.*, 2012). In mammalian cells μ ORF-encoded proteins include transcriptional regulators activating transcription factor 4 (ATF4), C/EBP homologous protein (CHOP) and a regulatory subunit of protein phosphatase 1, which can dephosphorylate eIF2 α (Sonenberg *et al.*, 2009).

eIF2 α phosphorylation at T169 in *T. cruzi* epimastigotes is induced by nutritional stress and mutation to alanine prevents full translation arrest impacting metacyclic differentiation (Tonelli *et al.*, 2011). eIF2 α phosphorylation also regulates protein synthesis in *Leishmania* (Chow *et al.*, 2011). Nevertheless, the role of eIF2 α availability and its phosphorylation on regulating translation remains largely uncharacterized in trypanosomes. Here we evaluate eIF2 α level, phosphorylation state and relevance to different stages of *T. cruzi*. eIF2 α is relatively decreased in trypomastigote forms, compatible with a low translation activity but is increased in amastigotes prior to differentiation into infective forms. To demonstrate the importance of this phosphorylation, we overexpressed wild type eIF2 α and eIF2 α ^{T169A} mutants and observed a large decrease in infectivity. Furthermore, alterations to the surface

proteome suggest specific changes to virulence-associated protein families, suggesting direct roles for eIF2 α in modulation of infectivity.

2. MATERIAL AND METHODS

The procedures used in this work were approved by the “Comite de Ética em Pesquisa da Universidade Federal de São Paulo” under protocol 2666291015. All methods were performed in accordance with the relevant guidelines and regulations approved by the Universidade Federal de São Paulo.

2.1. Parasites and cell cultures

Epimastigotes of *T. cruzi* (Y strain) were cultivated at 28 °C in autoclaved in LIT (liver infusion tryptose) medium supplemented with 1 μ g/ml hemin, 10% FBS, 2 mg/ml glucose, 59 mg/ml penicillin and 133 mg/ml streptomycin (Camargo, 1964). The parasites were diluted from 2×10^7 to 5×10^6 cells/ml to be kept in log phase growth. Epimastigotes overexpressing GFP fused to the N-terminal residue of histone H2b from *T. cruzi* (Ramirez *et al.*, 2000) and overexpressing TcelF2 α were both cultivated in the same medium with addition of 50 μ g/ml of Geneticin/G418 (Thermo Fisher Scientific).

Metacyclic-trypomastigotes were obtained from exponentially growing epimastigotes cultures (1×10^7 parasites/ml) after 4x dilution in Grace's insect medium (Thermo Fisher Scientific) previously adjusted to pH 6.2 with 2 N HCl. Metacyclic-trypomastigotes appeared after 7 days at 28 °C, and were purified by DEAE-cellulose columns (DE-52, Whatman) as described (Teixeira *et al.*, 1986). For metacyclogenesis quantification, late log phase cultured epimastigotes were centrifuged at 2000 g for 10 min, washed once in TAU medium (190 mM NaCl, 17 mM KCl, 0,035%, NaHCO₃, 2 mM CaCl₂, 2 mM MgCl₂, 1.5 mM sodium phosphate, pH 6.5) and suspended to 1×10^9 parasites/ml in TAU medium. Parasites were incubated for 2 h at 28 °C, diluted 100x in TAU3AAG medium (TAU containing 10 mM glucose, 2 mM aspartic acid, 50 mM glutamic acid and 10 mM proline) and maintained in 75 cm² tissue culture flasks for 5 days at 28 °C. The floating parasites were collected, attached

to glass coverslips, fixed with 50% cold methanol and stained with Giemsa reagent previously diluted 1:4 in tap water. Optical microscopy was used to count and identify epimastigotes and metacyclic forms by evaluating the position of kinetoplast relative to the nucleus.

T. cruzi tissue-culture derived trypomastigotes were obtained from supernatants of LLC-MK2 cells (Rhesus monkey kidney epithelial cells, ATCC CCL-7) cultivated in low glucose Dulbecco's Modified Eagle Medium (DMEM, Thermo-Fischer Scientific) supplemented with 3.7 g/l sodium bicarbonate, 10 % FBS, 59 mg/ml penicillin and 133 mg/ml streptomycin at 37 °C in a 5% CO₂ atmosphere. Alternatively, U-2OS cells (Human osteosarcoma cells, Banco de Células do Rio de Janeiro) were used in the same conditions. The cells were infected with metacyclic-trypomastigotes, or with trypomastigotes released from infected cells. The parasites were collected by centrifugation at 1500 g for 5 min, incubated for at least 60 min at 37 °C and trypomastigote enriched supernatants were collected and used for experiments.

Intracellular amastigotes were obtained from infected LLC-MK2 cells, first by removing the monolayer with trypsin, washing the cells in culture medium and suspending infected cells in 27 mM K₂HPO₄, 8 mM Na₂HPO₄, and 26 mM KH₂PO₄, pH 7.2. The cells were then lysed in a Potter-Elvehjem homogenizer, and free parasites were separated from cell debris by several rounds of centrifugation at 800 g for 2 to 5 min, before collecting the purified amastigotes by centrifugation at 2000 g for 5 min (Abuin *et al.*, 1999).

2.2. Parasite invasion and intracellular replication

To evaluate the progression of *T. cruzi* infection inside the mammalian cell, LLC-MK2 (or U-2OS) cells were seeded onto 13 mm glass coverslips in 24 well-plates and incubated at 37 °C. The next day, 5 x 10⁵ trypomastigotes from each lineage was diluted in 500 µl of culture medium and added to each well for incubation for 2 h. The wells were washed twice with PBS and the incubation continued for 2, 3 and 4 days. The coverslips were fixed in Bouin solution (5 % acetic acid, 9 % formaldehyde and

0.9 % picric acid) for 5 min and washed four or five times with PBS before staining for 1 h with Giemsa solution (previously diluted 1:10 in tap water and filtered in Whatman 2 paper). Glass coverslips were dehydrated in acetone, acetone with xylol, and only xylol before mounted in glass slides covered with Entellan mounting medium (Sigma-Aldrich). Coverslips were visualized under an optical microscope and the number of intracellular amastigotes was assessed in one hundred infected cells.

Alternatively, we employed a high content analysis of infection using U-2 OS human bone osteosarcoma cells, also maintained in low glucose DMEM and 10% FBS. Two thousand cells were suspended in 100 μ l medium, or 800 cells in 40 μ l, then seeded in 96 or 384 well-black plates with clear bottom (Greiner Bio-One), respectively. After 16-24 h at 37 °C, 50 μ l, or 10 μ l of a trypomastigote suspension in DMEM, containing the indicated concentrations of parasites obtained from infected cells were added respectively to the 96 or 384 well-plates. Two, three, or four days, after the infection the cells were fixed with 4 % PFA (paraformaldehyde) in PBS, stained with Draq5 (Biostatus) in the dark and at room temperature for 30 min.

The plates were then imaged by using a High Content Imaging System INCell (GE) at 20X magnification. Six images were acquired per well with thousands of cells per image. Images were analyzed with the High Content Analysis (HCA) software Investigator version 1.6 (GE) for identification, segmentation and quantification of host cell nucleus, cytoplasm and intracellular parasites. The HCA provided output data for all images as the total number of cells, total number of infected cells and the total number of intracellular parasites. The ratio of infected cells to the total number of cells was calculated and defined as the Infection Ratio for invasion assays and the mean number of amastigotes per infected cell was also calculated for replication assays.

2.3. *T. cruzi* eIF2 α constructions for overexpression

The coding gene of *T. cruzi* eIF2 α was amplified from genomic DNA of DM28c strain (TCDM_00323, <http://tritrypdb.org>) and inserted on pDONR221 (Thermo Fischer Scientific) Gateway vector before transferring to pDEST17 vector, as described

previously (Tonelli *et al.*, 2011). The substitution of serine 43 to alanine (S43A) was realized by two step PCR, initially generating fragments using Pfu polymerase (Thermo Fisher Scientific) and the primers TcelF2FX and TcelF2Rbg and eIF2SA and eIF2RH. All primer sequences are listed in table S1. Both fragments were gel purified and used as templates for the second PCR with TcelF2FX and eIF2RH to amplify the full version of TcelF2 α containing the mutated S43A. The substitution of the phosphorylation site at threonine 169 to alanine (T169A) was performed similarly, firstly utilizing the primers TcelF2 FX and eIF2T169ARev or eIF2T169AFow and eIF2RH, followed by a second PCR reaction to amplify a full version of TcelF2 α containing the mutated T169A using TcelF2FX and eIF2RH. To obtain a double-mutant (S43A/T169A), the TcelF2 α gene with mutated S43A was used as template for both PCR reactions employed to generate the T169A construct. The full length fragments were digested with EcoRI and HindIII and inserted on p33 vector (Ramirez *et al.*, 2000) previously digested with the same enzymes. The presence of the expected mutations was confirmed by sequencing reactions using primers p33SeqFow2 and eIF2RH.

To obtain parasite strains expressing modified eIF2 α , 4×10^7 epimastigotes were washed in 137 mM NaCl, 21 mM Hepes pH 7.0, 5.5 mM Na₂HPO₄, 5 mM KCl and 0.77 mM glucose and incubated with 15 μ g of circular plasmids diluted in the same buffer. The mixtures were submitted to electric pulses using 0.4 cm cuvettes in the Amaxa Nucleofactor (Lonza) set at program U-33 at room temperature. Pulsed parasites were then diluted in 5 ml of LIT medium containing 10 % de FBS and after 24 h Geneticin-G418 (Thermo Fisher Scientific) was added to 500 μ g/ml to obtain stable transformants. Chromosomal and episomal DNA were obtained from selected parasites, as described before (Medina-Acosta *et al.*, 1993), and submitted to sequencing reaction using the primer p33SeqFow2.

2.4. Generation of T169A replacement

The parasites of Y strain expressing Cas9 were generated by transfection with plasmid pTREX-Cas9-Neo (Lander *et al.*, 2015), selected and maintained in the same

medium supplemented with 200 µg/ml Geneticin G418. For transfection, 4×10^7 exponentially growing Cas9 epimastigotes were collected and centrifuged at 2,000 g for 5 minutes, parasites were washed in 1 ml of electroporation buffer (5 mM KCl, 0.15 mM CaCl_2 , 90 mM Na_2HPO_4 , 50 mM HEPES, 50 mM Mannitol) (Pacheco-Lugo et al., 2017) and resuspended in 100 µl of electroporation buffer, along with 50 µl containing 2 µg of purified sgRNA and 1 µmole of the donor sequence. The sgRNA was generated by PCR using the oligonucleotide sgelF2 (positions 578-597 of the eIF2 α ORF) and the common primer that recognizes the plasmid pUC sgRNA (Lander *et al.*, 2015). The sgRNA was prepared by in vitro transcription using the MEGAShortscript Kit (Thermo Fisher Scientific) according to the manufacturer instructions and purified by phenol/chloroform extraction and ethanol precipitation. The donor DNA was the oligonucleotide eIF2T169don designed to replace the Thr 169 and contains a BssHII site. Parasites were transfected in 2 mm cuvettes using AMAXA Nucleofactor II electroporator, with two pulses of X-014 program. Subsequently, parasites were transferred to bottles with fresh LIT medium supplemented with 10% fetal bovine serum and maintained at 28 °C for recovery. After 5 days, a new round of transfection was made. After the parasite recovery, the total DNA of the parasites were extracted and the region corresponding to eIF2 α using the primers eIF2S43DFow and eIF2RH. The PCR products before and after 3 h digestion with BssHII were analyzed in 1% agarose gels in TAE buffer and ethidium bromide staining. Parasite clones were obtained by serial dilutions in 96 well plates in LIT medium.

2.5. Western Blotting

Parasites were collected by centrifugation at 2000 g for 5 min and washed once in 50 mM Tris-HCl pH 7.5, 150 mM NaCl (TBS) containing phosphatase inhibitors (PhosStop, Sigma-Aldrich) and protease inhibitors (Complete, EDTA-free, Roche) as recommended by the manufactures. Cells were lysed by three cycles of freezing and thawing, centrifuged at 13000 g for 10 min at 4 °C and the supernatant containing the soluble proteins was collected. The amount of proteins was measured by Bradford assay (Bradford, 1976), mixed with SDS-PAGE sample buffer (Laemmli, 1970) and

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heated at 95 °C for 5 min. Alternatively, total extracts were prepared from TBS washed parasites by suspending the pellets directly on sample buffer followed by heat denaturation. Electrophoresis was performed in polyacrylamide gels containing SDS and the proteins transferred to nitrocellulose membranes by standard procedures. The membranes were stained with 0.5 % Ponceau S in 3 % acetic acid, blocked with TBS containing 0.1 % Tween 20 (TBS-T) and 5 % BSA for 1 to 12 h. For the TcelF2 α phosphorylation analysis in intracellular parasites, the membranes were cut separating the portion containing proteins with mass above 40 and below 60 kDa. Membrane pieces were incubated for 2 h with an affinity purified antibody that reacts with the phosphorylated T169 of TcelF2 α (Tonelli *et al.*, 2011) diluted in blocking buffer. The antibodies were re-purified by sequential affinity chromatography on peptides NH₃YTEI[^PT]RIRIRAIGKC-amide and NH₃YTEIRIRIRAIGKC-amide (GeneScript) both coupled to SulfoLink® Coupling Resin (Thermo Fisher Scientific). Elution from the first column was achieved by using 50 mM glycine pH 2.8 buffer, followed by neutralization by adding 2 M Tris-HCl pH 9.0 and the second column was only used to adsorb non-phospho-specific antibodies. Anti-*T. cruzi* eIF2 α antibodies that recognize the full protein was obtained by immunizing mice with the *T. cruzi* recombinant protein cloned in pET28a and expressed in *E. coli* BL21 as described previously (da Silva Augusto *et al.*, 2015).

Membranes were washed 3 times in TBS-T for 10 min, incubated for 1 h with anti-rabbit IgG peroxidase-conjugated antibody (Thermo Fischer Scientific) diluted 1:20,000 in TBS, and washed more three times in TBS-T for 10 min. Bound antibodies were detected by ECL (EMD Millipore) using an Odyssey Fc System (LI-COR Biosciences). The same membrane pieces were re-probed with an anti-TcelF2 α serum from mice immunized with a recombinant *T. cruzi* eIF2 α expressed in *Escherichia coli* BL21 using the pDEST17 vector and purification in Nickel-agarose column. The serum was diluted 1:2,000 in PBS containing 5 % non-fat dry milk. After 1 h incubation, the membranes were washed three times with TBS-T followed by 1 h incubation with anti-mouse IgG IRDye 800 (LI-COR Biosciences) 1:10,000 in PBS.

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Membranes were washed three times with TBS-T and bound antibodies detected as described above. Others immunoblots were performed with rabbit serum anti-Hsp70 diluted 1:10,000 (McDowell *et al.*, 1998), rabbit serum anti-TcelF5A diluted 1:10000 (Chung *et al.*, 2013), rabbit serum anti-BiP diluted 1:5000 (Bangs *et al.*, 1993), mice ascites for anti-gp82 3F6 monoclonal diluted 1:100 (Araya *et al.*, 1994) and mice ascites for anti-gp90 5E7 monoclonal diluted 1:100 (Franco *et al.*, 1993). Anti-*T. brucei* aldolase was prepared by immunizing rabbits with recombinant *T. brucei* aldolase, as described (Opperdoes *et al.*, 1977), all diluted in blocking buffer and incubated for at least 1 h and detected as described above. Pixel quantification was performed using Image Studio version 5.2 (LI-COR Biosciences).

2.6. Zymogram analysis

Trypomastigotes obtained from infected U2-OS cells and stored at -80°C until use. The parasites (3×10^7) were then thawed in 60 μ l of SDS-PAGE sample buffer without β -mercaptoethanol and incubated with 1 μ l Benzonase (Merck Millipore) and loaded in a 10% polyacrylamide gel containing 20 mg/ml gelatin. The electrophoresis was performed at 10 V/cm and at 4°C. The gel was then incubated for 1 h in 2.5% Triton-X100 at 37°C and then in 50 mM Tris-HCl pH 7.5, 200 mM, NaCl, 5 mM CaCl_2 , and 0.02% Brij-35 (v/v) for 12 h before staining with Coomassie Blue R250 and destaining in water/ethanol/acetic acid (4/4/1).

2.7. Parasite size quantification

Exponentially growing *T. cruzi* epimastigotes were collected by centrifugation at 2,000 g for 2 min, washed twice and suspended in PBS before addition to glass slides (Tekdon Inc) previously treated with 0.01 % poly-L-lysine (Sigma-Aldrich). After 5 min, unattached cells were removed, and the slides were incubated with PFA for 15 min. The slides were washed twice with PBS and mounted with Prolong Gold Antifade Reagent (Thermo Fisher Scientific). Differential interference contrast images containing at least one hundred parasites were acquired using an Orca R2 CCD camera (Hamamatsu) coupled to an Olympus BX-61 microscope equipped with a

x100 plan Apo-oil objective (NA 1.4) and the area of each parasite calculated after manually contouring each parasite using the Cell^M software (Olympus). Data was analyzed by non-parametric two-way Anova with Graph Pad version 6.

2.8. Flow cytometer analysis

Tissue culture derived trypomastigotes were collected and washed by centrifugation (2000 g, 5 min) and resuspended in PBS. The parasites were fixed by adding the same volume of a 4% *p*-formaldehyde in PBS, which were maintained at 4 °C until use. The fixed parasites were washed by centrifugation (2000 g, 5 min) in a swing bucket rotor twice with PBS and resuspended to 2×10^7 /ml. One hundred μ l of the culture supernatant of hybridoma mAb 3C9 (Andrews *et al.*, 1987) diluted 5X, the ascitic fluid of mAb Mest-1 (Suzuki *et al.*, 1997) diluted 100X in PBS, or Concanavalin-biotin (Sigma-Aldrich) diluted 100X was then added to 100 μ l of the fixed parasite suspension. After 1 hour incubation at room temperature, the suspension was centrifuged, washed once and resuspended in 100 μ l of PBS, before addition of the same volume of anti-mouse IgG-Alexa 488 (Thermo-Fisher Scientific) or Streptavidin-Phycoerythrin (BD Biosciences). The samples were then incubated 30 min, washed, resuspended in 250 μ l of PBS and fixed with the same volume of 4% *p*-formaldehyde in PBS. The samples were then analyzed in Accury C6 flow cytometer (BD Biosciences).

2.9. Polysome Profiling

At least 1×10^9 exponentially growing epimastigotes, before or after pre-incubation with TAU medium for 2 h were treated with 100 μ g/ml cycloheximide at 28 °C for 10 min, followed by ice cooling for additional 10 min. The cells were then collected by centrifugation at 2,000 g for 5 min at 4 °C, washed in ice-cold PBS containing the same concentration of cycloheximide and suspended in approximately 300 μ l of ice-cold Buffer A (10 mM Tris-HCl, pH 7.4, 300 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol and 100 μ g/ml cycloheximide). A drop of Triton X-100 was added to the lateral side of tube to reach 1 % (w/v) and the tubes were repeatedly mixed by inversion for about 3-5

min. The lysates were centrifuged at 6000 g for 3 min at 4 °C and the supernatants were transferred to a new tube. Stocks of heparin at 100 mg/ml and NaCl at 5 M were added to 1 mg/ml and 150 mM, respectively. Samples containing the equivalent of ten absorbance units at 260 nm were then loaded on the top of a 7 % to 47 % sucrose gradient in Buffer A, prepared using a Gradient Master (Biocomp). The tubes were centrifuged at 250,000 g for 2.5 h in a Beckman SW41 rotor at 4 °C. The gradient was collected from the top by injecting the bottom with a continuous flow of 60 % sucrose at 1 ml/min using an Econo Gradient Pump (Bio-Rad) and the fractions were monitored by reading the absorbance at 254 nm.

2.10. Parasitemia and immunosuppression analysis

BALB/c female mice (8 to 10 weeks old) were obtained from the CEDEME animal facility at UNIFESP and were maintained under pathogen-free conditions in individually ventilated cages, experiencing a 12 h light/dark cycle with access to food and water *ad libitum*. Trypomastigotes (5×10^5) from the indicated lineages were injected intraperitoneally in five animals per group. Blood parasitemia was quantified by counting motile parasites in 5 μ l fresh blood drawn from lateral tail veins every two days starting on day 1. Forty days after infection, each animal received 100 mg of cyclophosphamide (Genuxal, Baxter) per kg of body weight every three days (Huyan *et al.*, 2011). The parasitemia was then verified every 3 to 5 days and animal survival was recorded daily.

2.11. Proteomic analyses

For proteomic analysis parasites were maintained in LIT-10% FBS up to 1×10^7 ml in five independent cultures at 28 °C performed in different weeks. The parasites were collected by centrifugation at 2000 g, 10 min, washed once in PBS and stored at -80 °C. The samples containing 2×10^7 parasites resuspended, boiled in 20 μ L of SDS-PAGE sample buffer containing 5 mM DTT and loaded in precast Novex Value 4-12% Tris-Glycine gels. The electrophoresis was performed in 50 mM MOPS-50 mM Tris Base, 3.5 mM SDS, 1 mM EDTA at 100 V for 10 min. The migration portion of each

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lane were cut, and the gel pieces were fixed for 10 min at room temperature in 1 volume of 40% ethanol, 10% acetic acid. The fixative was removed, and the fixation repeated twice more. The bands were kept frozen at -80 °C until processing for mass spectrometry analysis. The gel slices were submitted to reductive alkylation and in gel tryptic digest using routine procedures. The eluted peptides were then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS²) on a ultimate3000 nano rapid separation LC system (Dionex) coupled to a Q Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo-Fisher Scientific). Spectra were processed using the intensity-based label-free quantification (LFQ) method of MaxQuant (Cox *et al.*, 2008) searching the *T. cruzi* YC6 annotated protein database (release 46) from TriTrypDB (Aslett *et al.*, 2010). Minimum peptide length was set at six amino acids, isoleucine and leucine were considered indistinguishable and false discovery rates (FDR) of 0.01 were calculated at the levels of peptides, proteins and modification sites based on the number of hits against the reversed sequence database. When the identified peptide sequence set of one protein contained the peptide set of another protein, these two proteins were assigned to the same protein group. The LFQ data were analyzed using the Perseus software (Tyanova *et al.*, 2016). A student's *t*-test comparing the control sample group to the respective modified strain generated -log₁₀ *t*-test *P*-value plotted versus *t*-test difference to generate a volcano plot. Enrichment analysis was performed using a Fisher's exact test in Perseus with a Benjamin–Hochberg false discovery rate of 0.02. Proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (Vizcaino *et al.*, 2016) with the dataset identifier PXD018761.

2.12. 5'UTR, μ ORF and IRES detection.

The full genome of *T. cruzi* YC6 sequenced and annotate by Dr. Wei Wang and Prof. Rick Tarleton from the Center for the Tropical & Emerging Global Diseases, University of Georgia was downloaded from <https://tritrypdb.org/tritrypdb/> (Aslett *et al.*, 2010) (version 46) and the RNAseq corresponding to the four parasite stages was kindly provided by Prof. Rick L. Tarleton and Dr. Wei Wang. The 5'UTR was obtained

using the UTRme package (<https://github.com/sradiouy/UTRme.git>) using default parameters. μ ORFs were annotated as in (Radío *et al.*, 2020). Briefly, Open reading frames (ORF) in the 5'UTRs were obtained using the getORF tool (Rice *et al.*, 2000).

μ ORFs in which the coding sequences start in the 5'UTR region and end within the main CDS (out of frame with respect to the main CDS AUG) were defined as overlapping μ ORFs (μ ORFo) while the ones contained entirely in the 5'UTR were defined as non-overlapping μ ORF (μ ORFno), in this case the frame with respect to the main CDS AUG was not considered. Only μ ORFs with this characteristics were considered in the analysis: 1) AUG start codon; 2) at least 15 nucleotides from the μ AUG to the 5' end of the mRNA; 3) a maximum distance of 50 nucleotides between the stop codon of the μ ORF and the AUG of the main CDS; 4) minimum length of 5 amino acids. In the case of the μ ORFo the requirements were: 1) start with AUG; 2) a minimum length of three amino acids before the AUG of the main CDS. The IRES sequence were predicted based on the best-score 5'UTR sequences, using IRESpy—Prediction of IRES by XGBoost (<https://irespy.shinyapps.io/IRESpy>). The data was analyzed and visualized using the Orange3 Data Mining (<https://orange.biolab.si>).

3. RESULTS

3.1. EIF2 α expression is decreased in non-proliferative *T. cruzi* life stages.

To characterize alterations in eIF2 α across multiple developmental stages of *T. cruzi* Western blotting was used with an antibody generated against bacterially expressed *T. cruzi* eIF2 α . Ten micrograms protein from soluble extracts obtained from replicative epimastigotes (Epi) and intracellular amastigotes (Ama) and non-proliferative but infective metacyclic-trypomastigotes (Meta) and tissue culture-derived trypomastigotes (Trypo) were subjected to electrophoresis and blotting (Figure 1A). Both Meta and Trypo possess reduced levels of eIF2 α compared to proliferative forms (Figure 1B) and were specific as similar decreases were apparent relative to Hsp70

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and BiP, cytosolic and ER chaperones respectively (Figure 1C and D). We also noticed diminished expression of eIF2 α relative to eIF5A in metacyclic-trypomastigotes (Figure 1E), consistent with a specific decrease in proteins involved in translation recently described in metacyclics (Schuller *et al.*, 2017). Therefore, we suggest that down-modulation of eIF2 α expression in infective forms likely relates to diminished translational activity and may also reflect altered protein expression patterns in proliferating versus infective *T. cruzi* stages.

3.2. eIF2 α phosphorylation increases in the intracellular replication cycle prior to differentiation into trypomastigotes

Trypomastigotes enter mammalian cells by forming a parasitophorous vacuole, and from 4 to 12 h, depending on the host cell and parasite strain, most parasites exit the vacuole and transform into amastigotes (Rubin-de-Celis *et al.*, 2006). Amastigotes start dividing in the host cell cytosol 24 hours after invasion and in about four days occupy the entire cytosol and transform into trypomastigotes, which are released by host cell rupture, usually after five days of infection. To further investigate how and whether a decrease in translation in trypomastigotes is related to eIF2 α phosphorylation, we examined intracellular amastigotes collected two, three and four days after infection by Western blotting with an antibody against phosphorylated T169 of eIF2 α ^{T169P} relative to total eIF2 α and Hsp70. eIF2 α ^{T169P} increased from 72 hours, peaking at 96 hours after infection, the last day before differentiation into trypomastigotes (Figure 2A). Quantitative analysis confirmed these observations and indicate that released trypomastigotes have decreased eIF2 α ^{T169P} relative to both total eIF2 α (Figure 2B) and Hsp70 (Figure 2C) three and four days post-infection. Therefore, increased levels of eIF2 α ^{T169P} occur in amastigotes, probably related to differentiation, but concomitant with decreased total eIF2 α and translation.

3.3. Generation of parasite lines overexpressing non-phosphorylatable eIF2 α and parasites.

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eIF2 α contains an N-terminal extension adding 118 amino acids in *T. cruzi*, such that T169 corresponds to S51 in other eukaryotes (Figure 3A) and is involved in regulation of global protein translation in *T. cruzi* epimastigotes (Tonelli *et al.*, 2011). To determine if T169 phosphorylation also affects control of protein synthesis and differentiation of *T. cruzi*, and specifically in mammalian stages of the parasite, we overexpressed eIF2 α in a similar approach as used by Tonelli *et al.* (2011) but without an epitope tag. Four different epimastigote lines were produced: a wild-type version (WT), a version with serine 43 mutated to alanine (S43A), a version with T169 mutated to alanine (T169A) and a version with both mutations (S43A/T169A). A control containing the green fluorescent protein (GFP) expression fused to histone H2B was also prepared as described previously (Ramirez *et al.*, 2000). The epimastigote lines had over three-fold overexpression of eIF2 α , which was stable at least until 21 days without drug selection (Figure S1A and S1B). Interestingly, cells from these lines were smaller (Figure S1C and E) and importantly global translation was not completely abrogated in comparison with the wild type line incubated in starvation conditions (Figure S2A), as shown previously (Tonelli *et al.*, 2011).

We also generated parasites with the eIF2 α ^{T169A} mutation using CRISPR/Cas9 to specifically mutate both endogenous loci. Cells expressing a Cas9-GFP fusion were transfected with an appropriate guide RNA and donor oligonucleotide in which the T169A mutation and a BssHII site were inserted. This was prepared by targeting the single guide RNA to the eIF2 α ORF, just after the T169 coding sequence (AGG). The repairing was made using a donor containing the sequence GCGCGC (BssHII site) to code for the amino acids A and R. After two rounds of transfection, more than 95% of the parasites contained the insertion as evaluated by PCR and BssHII restriction (Figure 3B) and the correct mutation validated by sequencing. The parasites were cloned by limiting dilution and a clone displaying full BssHII digestion selected. Digestion with BssHII of the PCR product corresponding to the eIF2 α gene showed full digestion, confirming the absence of parasites without mutation (Figure 3B). Moreover, lysates from the eIF2 α ^{T169A} clone were not recognized by anti-phospho-

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eIF2 α antibodies, even after nutritional stress (TAU), a condition that increases phosphorylation of eIF2 α (Figure 3C). Importantly, protein levels of eIF2 α were similar in both cell types and we always detected some eIF2 α phosphorylation in wild type parasites. The eIF2 α^{T169A} overexpression parasites also showed less efficient translation arrest compared with the eIF2 α^{T169A} Cas9 strain as was the case of the overexpressors containing the mutation (Figure S2B). The epimastigotes lines were also used to obtain metacyclic-trypomastigotes, which infected mammalian cells and produced new rounds of cell-derived trypomastigotes. In the eIF2 α^{T169A} overexpressing lines, these trypomastigotes continued to exhibit higher levels of eIF2 α in relation to Hsp70 and aldolase compared to wild type parasites indicating that the overexpression phenotype remained stable despite differentiation (Figure S1E and F).

3.4. Absence of eIF2 α phosphorylation affects protein expression in *T. cruzi*

Epimastigotes and metacyclic-trypomastigotes derived from eIF2 α^{T169A} overexpressor lines expressed increased amounts of the trans-sialidase family members gp82 and gp90 (Figure S3A), both of which are preferentially expressed in metacyclic-trypomastigotes (Yoshida *et al.*, 1990; Araya *et al.*, 1994). Quantitative analysis indicated a three-fold increase in gp90 expression for eIF2 α^{T169A} and eIF2 $\alpha^{S43AT169A}$ cells compared to GFP, WT or eIF2 α^{S43A} parasites (Figure S3B). No differences were observed for antibodies that recognized mucin-like glycoprotein in these epimastigotes. In contrast eIF2 α^{T169A} parasites obtained by CRISPR did not express gp90 in epimastigotes (Figure S3C), indicating that overexpression of eIF2 α^{T169A} differently affected parasites compared to mutation and retention of endogenous expression levels.

To characterize these changes in more detail CRISPR/Cas9 parental and eIF2 α^{T169A} mutant cells were analyzed by LC-MSMS. 4301 protein groups were identified, representing approximately ~30% of the *T. cruzi* proteome (Table S2, protein groups). Considering only robust data, (applying a cut-off $-\log_{10} P$ -value > 1; Table S2 increased and decreased Log p>1, see methods) calculated using 5 control independent samples and 4 independent T169A samples (Table S2, summary), the

abundance of 217 protein groups was decreased and the abundance of 205 were increased (Figure 3D). As *T. cruzi* genome annotation remains rather poor, we grouped these protein groups by function based on possession of Interpro domains and similarities to annotation of syntenic genes in other trypanosomatids, mainly *T. brucei* (Figure 4 and Table S3). We found that the mutant had decreased levels of surface proteins, glycoprotein synthesis enzymes, redox enzymes, cell cycle control, cytoskeleton, several phosphatases, ADP-ribosylation and lipid metabolism. In contrast, absence of eIF2 α phosphorylation produced cells with more proteins involved in amino-acid metabolism, several chaperones, chromatin structure and transcription. Furthermore, trans-sialidase gene family members are significantly enriched in the decreased cohort ($-\log P > 0.5$ cutoff) with an enrichment factor of 2.5 and a false discovery rate (FDR) $< 2\%$ (Table S4). It is also particularly relevant that the eIF2 α kinase similar to PERK (Tck2) increased about seven times in the mutant cells, suggesting an adaptation by these mutants to rectify the decrease in phospho-eIF2 α^{T169} by increased kinase activity. Protein biosynthesis and surface export pathways appear impacted and several activities related to transcription and chromatin state are affected.

As genes containing micro-Open Reading Frames (μ ORF) are preferentially translated upon eIF2 α phosphorylation (Andreev *et al.*, 2015), we generated the 5' untranslated regions and 3'UTR of the mRNAs of *T. cruzi* YC6 genome using the UTRme software, specifically developed for trypanosomatids (Radio *et al.*, 2018) (Table S4) and μ ORFs with repressive characteristics were annotated (see Materials and Methods section for details). When we selected the proteomic hits within high confidence prediction values ($-\log_{10} P\text{-value} > 1$), we found no correlation between the 5' UTR length and increased protein expression (Figure S4A). Overlapping μ ORFs were slightly enriched in the downregulated proteins cohort with an enrichment factor of 1.7 (FDR $< 2\%$) (Table S4).

As the codon adaptation index (CAI) has been linked to protein levels in trypanosomes (Jeacock *et al.*, 2018), decreased eIF2 α phosphorylation could affect

less abundant proteins, i.e. those with low MS/MS counts. Genes with high CAI are more expressed in epimastigotes (Figure S5A). However, abrogation of the eIF2 α phosphorylation showed no correlation with the cohorts of differentially expressed proteins (Figure S5B). The data of CAI for all CDS detected in *T. cruzi* is shown in Table S5.

3.5. Impaired phosphorylation of eIF2 α decreases parasite infectivity and replication in mammalian host cells

Next, we investigated the importance of eIF2 α to mammalian cell and animal infectivity by using eIF2 α mutant trypomastigotes. We used U-2 OS human osteosarcoma and LLC-MK2 epithelial kidney cells as *in vitro* infection models (Moon *et al.*, 2014). Initially, we measured infection ratio i.e. number of infected relative to total cells, using different multiplicities of infection (MOI) of parental, eIF2 α overexpressors and eIF2 α ^{T169A} mutants. eIF2 α ^{T169A} parasites had a significantly decreased infectivity compared to control parasites even at different MOI (Figure 5A). We detected a significant increase of amastigotes replication in cells overexpressing wild type eIF2 α compared to control cells. eIF2 α ^{T169A} overexpressor lines had a decreased proliferation rate (Figure 5B), which was clear in stained cultures (Figure S6A) and across different MOI (Figure S6B to S6D). Similar results were observed when infecting LLC-MK2 cells, showing a reduced number of intracellular amastigotes in cells infected with both eIF2 α ^{T169A} lineages (Figure 5C). A similar decrease in intracellular proliferation could be observed just after two and three days of infection (Figure S6E and F, respectively). Importantly, fewer trypomastigotes were released from LLC-MK2 cells infected with eIF2 α ^{T169A} mutants compared to control lines (Figure 5D), expected, due to their decreased invasion and/or growth rate.

We also examined the impact of eIF2 α mutation by CRISPR/Cas9. Mutated epimastigotes differentiate less into metacyclic-trypomastigotes (Figure 5E) and trypomastigotes released from infected mammalian cells showed decreased invasion of U-2 OS cells (Figure 5F). Furthermore, the mutant amastigotes proliferated more slowly and less trypomastigotes were released from infected (Fig. 5G and H),

confirming that the absence of eIF2 α phosphorylation contributed to compromised infectivity in an *in vitro* model.

3.6. Parasites containing non-phosphorylatable eIF2 α are less virulent in mice

Overexpressor eIF2 α^{T169A} trypomastigotes yield reduced levels of blood parasitemia in mice compared to parental or to parasites overexpressing wild type eIF2 α (WT) (Figure 6A). This decrease was even more pronounced in the second peak of parasitemia, indicating that the animals were more efficiently controlling the infection caused by eIF2 α^{T169A} parasites. As animals survived the acute phase, to determine if they remained infected, mice were immunosuppressed with cyclophosphamide. Parasites appeared later, and with lower parasitemia in the blood of mice when infected with eIF2 α^{T169A} overexpressing parasites compared to the control lines (Figure S7A). Indeed, this resulted in delayed mortality in the animals infected with eIF2 α^{T169A} overexpressors (Figure 7B). Together, these results indicate that overexpression of non-phosphorylatable eIF2 α^{T169A} modulates *T. cruzi* infectivity.

3.7. Mutant trypomastigotes have a distinct surface composition

As many surface molecules are known to be important for infection, we collected trypomastigotes released on the first and second day from infected cells and compared the major surface proteins between mutant and control parasites. Released parasites were fixed in *p*-formaldehyde and stained with monoclonal mAb 3C9, which recognizes sialylated mucin-like glycoproteins and with concanavalin A, which recognizes surface glycoproteins containing N-linked oligomannose chains, found in the large family of *trans*-sialidase proteins (Mucci *et al.*, 2016). Mutated parasites express higher amounts of both surface markers compared to control parasites (Figure 7A-D). In contrast, galactofuranose epitopes in glycosyl phosphatidylinositol (GPI) glycolipids recognized by mAb Mest-1 (Suzuki *et al.*, 1997), found important for *Leishmania* invasion (Suzuki *et al.*, 2002), were less exposed (Figure 7E and D). Mutated parasites also presented decreased amounts of the surface protease

metalloprotease GP63, also a (Cuevas *et al.*, 2003; Kulkarni *et al.*, 2009) as detected by zymogram analysis, indicating significant changes on the parasite surface.

4. DISCUSSION

Here we evaluated the expression and phosphorylation state of *T. cruzi* eIF2 α during all phases of the parasite life cycle, a key regulator of the translational apparatus. Total protein levels of eIF2 α decreased in infective and non-proliferative trypomastigotes compared to both proliferative epimastigotes and amastigotes, while phosphorylated eIF2 α increased during amastigote infection prior to the generation of trypomastigotes. When non-phosphorylatable eIF2 α was overexpressed or endogenous eIF2 α was replaced by the same mutated form parasites differentiate less efficiently and have lower infectivity. These changes occurred in parallel with alterations in protein expression, indicating a key role for eIF2 α phosphorylation in life cycle progression as well as implicating the impacted proteins as players in this process.

Reduced eIF2 α levels in trypomastigotes is compatible with previous studies showing decreased translation in infective forms (Avila *et al.*, 2003; Smircich *et al.*, 2015), in parallel to a general transcriptional reduction (Elias *et al.*, 2001). As eIF2 α phosphorylation is increased in intracellular amastigotes this event could trigger translational decrease and induce differentiation into trypomastigotes, as observed in the transformation of epimastigotes to metacyclic-trypomastigotes (Tonelli *et al.*, 2011). A similar mechanism is triggered for *Leishmania* when parasites infect macrophages and transform into amastigotes (Cloutier *et al.*, 2012), but is in contrast to *T. brucei* in which the equivalent eIF2 α mutation does not prevent differentiation into stumpy forms (Avila *et al.*, 2016), a form with reduced translation levels (Brecht *et al.*, 1998). *Toxoplasma gondii* elevated levels of phospho-eIF2 α are found in latent bradyzoites, the non or slow replicative form (Joyce *et al.*, 2010). Therefore, control of translational arrest likely relies on distinct mechanisms in different lineages.

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The pattern of protein expression in epimastigotes growing exponentially in rich medium is significantly altered when eIF2 α phosphorylation is blocked. Under these conditions, eIF2 α phosphorylation is present and suggests that translational initiation is constantly modulated in these cells. The decrease of surface protein expression, enzymes involved in maintaining redox homeostasis and control of cell cycle, likely explain both decreased differentiation and infectivity. eIF2 α phosphorylation clearly alters expression of specific trypomastigotes factors, and these likely include *trans*-sialidases, expressed in the final days of intracellular replication and prior to appearance of trypomastigotes (Frevert *et al.*, 1992; Abuin *et al.*, 1999). The importance of precise regulation of protein expression at this point in the life cycle has been noted in the highly attenuated CL-14 clone of *T. cruzi*, which is significantly compromised in development of trypomastigotes (Belew *et al.*, 2017). Reduced levels of enzymes such as peroxidases and oxidoreductases could also impair parasite fitness in mammalian cells as both invasion and proliferation are largely diminished.

We did not observe correlations with predicted 5'UTR length and the variation of protein levels in exponentially growing epimastigotes. Also, there was no strong correlation between the presence of μ ORFs and proteins downregulated in epimastigotes, which, based on other studies, would be expected as a consequence of eIF2 α phosphorylation. However, a slight overrepresentation of overlapping μ ORFs was indeed observed within the cohort of decreased proteins in eIF2 α T169A mutant. This type of sequence has been suggested to exert a significant repression in translation efficiency of the main CDS in *T. cruzi* (Radío *et al.*, 2020). Interestingly, TcMucII, surface protease GP63 and the UDP- β -galactofuranosyl-transferase surface proteins related to virulence in trypomastigotes were shown to contain μ ORF (Figure S4C) and were found decreased in trypomastigotes. The decreased amounts of β -galactofuranose in glycolipids and GP63 protease could explain the decreased invasion capacity of the eIF2 α mutations (Suzuki *et al.*, 2002; Kulkarni *et al.*, 2009). Also, the high recognition levels of sialylated mucins and concanavalin A in mutated parasites could, therefore, reflect their differential expression. One possibility is that

translation arrest could potentiate their expression by excluding them from RNA stress granules (Aulas *et al.*, 2017). The presence of specific RNA binding proteins could then direct the mRNAs for storage or degradation (Hentze *et al.*, 2018). In fact, several RNA binding proteins has been shown to control the fate of the different mRNAs in *T. cruzi* (Romagnoli *et al.*, 2020).

Specific changes in expression in trypomastigotes overexpressing eIF2 α mutants also included increased surface gp90. Gp90 is GPI-anchored (Guther *et al.*, 1992) and belongs to the sialidase family (Franco *et al.*, 1993) and it is mainly expressed in metacyclic-trypomastigotes (Araguth *et al.*, 1988). Gp90 inhibits cell invasion by metacyclic forms (Malaga *et al.*, 2001) by down-modulating lysosomal sequestration during parasite entry (Rodrigues *et al.*, 2017). Therefore, the presence of gp90 in trypomastigotes bearing the eIF2 α mutants offers additional reasonwhy, in the overexpressor lineages, we found infective parasites. However, mutation of endogenous eIF2 α did not impact on gp90, suggesting that dysregulation due to eIF2 α may require increased levels of the protein rather than simply loss of phospho-forms. Nevertheless, the phenotypes of both types of mutants are similar, with parasites becoming less infective and proliferative in mammalian cells. An excess of non-phosphorylatable eIF2 α could act to inhibit eIF2 α -kinases, which are crucial in many parasites (Zhang *et al.*, 2013; da Silva Augusto *et al.*, 2015; Rao *et al.*, 2016), and in support of this model we found increased eIF2 α kinase in the Cas9 mutated cells which is potentially a compensatory mechanism. Significantly, non-phosphorylated eIF2 α interacts more strongly with eIF2B (Bogorad *et al.*, 2017), which recycles GDP-eIF2 to GTP-eIF2 necessary to form the trimeric complex and hence indicating that the mutant forms likely block the function of the entire complex.

In summary, we demonstrate that different developmental stages of *T. cruzi* are accompanied by changes in both overall levels of eIF2 α and specific phosphorylation at threonine 169. The development of infective non-proliferative trypomastigotes that occurs in mammalian cells occurs in parallel to the phosphorylation of eIF2 α followed by its decreased expression. When phosphorylation is prevented, the invasion and

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multiplication of intracellular parasites is reduced, most likely a consequence of altered translational activity before transformation in infective forms. Trypomastigotes shows low levels of eIF2 α that remains unphosphorylated. Therefore, eIF2 α phosphorylation is an additional level to regulate protein expression in *T. cruzi* especially during differentiation to infective forms of the parasite.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTION

F.C.M., performed the experiments shown in Fig. 1, 2, 3A, 5A-D, 6, S1A-B, S1A and B, S1E and F, S2A, S3A and B, S6, S7; P.B-C, cloned and characterized the Cas9-T69A clone and performed the experiments shown in 3B and C, 5E-H; A.M.M., generated the Cas9-T169 and S2B and S3C; M.A. helped to isolate the Cas9-T169

line and performed the experiments in 3B, S1C, S3C and S1D; M.Z performed bioinformatic analysis of the proteomic data that generated Fig. 3d and Tables S2-3; M.C.F. helped in planning the proteomic analysis; R.S. calculated and analyzed the μ ORF; P.S. provide the strategy to determine the 5'UTRs; S.S performed the experiments of Fig. 7, gene identification and correlation analysis; S.S., F.C.M, P. B-C., A.M.M, P.S. M.Z. and M.C.F. planed, analyzed data and wrote the manuscript.

Figure Legends

FIGURE 1 Reduced eIF2 α expression in non-proliferative forms of *T. cruzi*. (A) Western blotting of soluble extracts containing 10 μ g of protein per lane was obtained from the indicated amounts of epimastigotes (Epi), metacyclic-trypomastigotes (Meta), intracellular amastigotes (Ama) and tissue-cultured derived trypomastigotes (Trypo) forms. The membranes were probed with antibodies to eIF2 α , Hsp70, BiP and eIF5A. Means of eIF2 α signal relative to parasites numbers (B), BiP (C) Hsp70 (D) and eIF5A (E) were calculated from duplicate experiments and the symbols indicate the values obtained in each experiment. Considering the eIF2 α versus all other proteins simultaneously, a significant difference was observed ($p = 0.0278$, $n = 6$) using Welch two tailed t-test.

FIGURE 2 Kinetics of eIF2 α phosphorylation during amastigote development. (A) Total cellular extracts obtained from 5×10^6 intracellular amastigotes collected 2, 3 or 4 days post-infection and from 2×10^7 trypomastigotes released in the supernatant (Trypos) were used in Western blotting. The membranes were probed with antibodies to total eIF2 α protein, antibodies to the phosphorylated form of eIF2 α at threonine 169 (T169^P) and anti-Hsp70. Images from two independent experiments are shown (Exp 1 and Exp 2). (B) Pixel signal quantification of phosphorylated T169^P relative to values obtained for total eIF2 α normalized to the values at day 2 post-infection. (C) Values of total eIF2 α (open symbols) and T169^P (closed symbols) relative to Hsp70 levels. Values show points of the different experiments and mean \pm standard deviation values of four independent experiments. * indicates $p < 0.05$ in relation to 2 days post-

infection values calculated using two-way ANOVA considering the means of individual experiments followed by Dunnett's correction.

FIGURE 3 T169 mutation in eIF2 α deregulates stage specific expression. (A) Diagram comparing *T. cruzi* and human eIF2 α . The arrows indicate the phosphorylation site at threonine 169 (T169) of *T. cruzi* eIF2 α and serine 51 (S51) of the *Homo sapiens* eIF2 α . The black boxes correspond to S1 domain, responsible for interaction with the ribosomes. (B) Agarose gel (0.8%) stained with ethidium bromide of the PCR product corresponding to a fragment of the eIF2 α gene obtained from the indicated epimastigote lines, untreated (-) or treated (+) with BssHII enzyme. The size markers are indicated on the left side of the gel in base pairs (bp). (C) Western blotting of epimastigotes ($\sim 3 \times 10^6$ per lane) from the indicated lines pre-incubated for 6 h in LIT medium or in TAU medium. The top panel corresponds to membranes incubated with antibodies to the phosphorylated eIF2 α (T169^P) followed by mouse anti-eIF2 α developed using IRDye680 and IRDye800 respectively. (D) Volcano plot showing the difference in expression of the mutant relative to control strains identified by mass spectrometry using five independent samples of controls and five independent samples of Cas9-T169A epimastigotes maintained in LIT medium. The samples were run on SDS-PAGE gels and analysed by LC MS/MS as described in Methods. The volcano plot shows the $-\log_{10}$ -transformed *t*-test *P*-value versus the *t*-test difference (difference between means) for each protein group. High confidence cohorts (with $-\log_{10}$ *t*-test *P*-values above 1) used in the enrichment analysis are in highlighted in color.

FIGURE 4 Unique gene types are decreased and increased in eIF2 α mutated epimastigotes. Different functional categories were assigned to protein groups quantified by the MS/MS analysis and plotted against the *t*-test difference (considering the number of hits across the respective difference interval (Size of horizontal large blue bars). These functional categories were obtained by direct comparison with annotations for syntenic genes of other trypanosomatids and Interpro domains. The box plot was generated by the Orange3 software and the mean (yellow) and median

values (blue vertical lines) estimated for each case. The thick blue line represents the limit of significant variations and the dotted line the observed end values.

FIGURE 5 Non-phosphorylatable overexpression of eIF2 α impairs invasion, amastigote replication and trypomastigote release. (A) The infection ratio was calculated based on the number of infected cells per total number of U-2 OS upon incubation with the indicated trypomastigote lines [(non-transfected, NT, black boxes); WT (green boxes); and T169A (red boxes)] overexpressors for 2 hours at different multiplicity of infection (MOI). The cells were washed and fixed after 3 days to allow better visualization by the HCA method. The values are means (white traces), 25 and 75% percentiles (colored boxes) and min and max values (deviation lines) of four independent experiments, each one measured in triplicate. * indicates $p < 0.05$ and ** $p < 0.01$ calculated using the Two-Way ANOVA in relation to NT parasites. ## indicates $p < 0.01$ and ### $p < 0.001$ also calculated by Two-Way ANOVA, using Tukey test correction, in relation to WT overexpressors. (B) HCA of intracellular amastigotes multiplication plotted as the number of amastigotes per infected U-2 OS cells 2, 3 and 4 days after infection with trypomastigotes (MOI = 40) of the indicated lineages. The values are means \pm standard deviation of three independent experiments and * indicate $p < 0.05$, ** indicate $p < 0.01$ and *** $p < 0.001$ calculated using the Two-Way ANOVA, following Tukey test correction, at the third and fourth day of replication. (C) The graph shows the number of amastigotes present in each cell calculated from 100 infected LLC-MK2 cells determined at 4 days after infection with the indicated Trypo lineages (MOI = 10). The values were determined by optical microscopy after Giemsa staining, as described in Methods. The values are represented as in (A) and * indicates $p < 0.05$ and ** $p < 0.01$, both calculated using the one-way ANOVA considering the relation with NT parasites. # indicates $p < 0.05$, ## indicates $p < 0.01$ and ### indicates $p < 0.001$ calculated using the one-way ANOVA in relation to WT overexpressors. (D) LLC-MK2 cells were infected with the same number of trypomastigotes of each indicated cell lineages and the parasites released every day on the supernatant was assessed by counting by using a

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Neubauer chamber from 4 to 10 days after infection. The values are means \pm standard deviation of three independent experiments and the differences are indicated with * ($p < 0.05$) in relation to NT parasites and # ($p < 0.05$) in relation to WT overexpressors, both calculated using Student's t-test. (E) Metacyclic-trypomastigotes observed 7 days after the inoculation of exponentially growing epimastigotes ($1 \times 10^7/\text{ml}$) in Grace's medium containing 10% FBS (initial concentration of epimastigotes was $3 \times 10^7/\text{ml}$). (F) Trypomastigotes ($1 \times 10^6/\text{ml}$ in 1 ml) were added to U-2 OS (MOI=10) in 24 well plates containing glass coverslips. After 2 h at 37°C , the parasites were removed, and the number of internalized parasites in duplicates were counted after Giemsa staining using two wells for each condition. In another set, the cells were further incubated in DMEM-10% FBS and stained after 72 h (G). Numbers in E to G are mean and \pm standard deviation of three independent experiments. * shows $p < 0.05$ using t-Student test. (H) LLC-MK2 cells (5×10^5) in 75 cm^2 were infected with trypomastigotes (5×10^6 – MOI=10) for 24 h. The parasites were replaced by fresh medium. The amount of released trypomastigotes of each line was then counted in the culture supernatant and the values shown are cumulative numbers of released parasites.

FIGURE 6 EIF2 α phosphorylation affects infectivity in mice. (A) Mice were intraperitoneally infected with trypomastigotes of the indicated overexpressor lines and the number of parasites in the blood was followed as described in Methods. The plotted values are means \pm standard error of two independent experiments using ten mice for each group. * indicates $p < 0.05$ in relation to NT parasites and # $p < 0.05$ in relation to WT overexpressors, both calculated using Student's t-test. (B) Forty days after infection, mice started to be immunosuppressed with cyclophosphamide, as described in Methods. The plot shows the days after immunosuppression and the survival was followed for NT ($n=7$), WT overexpressors ($n=6$) and T169A overexpressors ($n = 5$) infected mice showing different survival medians for each lineage.

FIGURE 7 Effect of T169A replacement on the expression of surface proteins of trypomastigotes. Trypomastigotes obtained from infected U-2 OS cells at the 6th and

7th day of infection of control cell line (parasites containing the Cas9 gene, blue lines) and the parasite line in which eIF2 α was replaced (Cas9-T169A, red lines) were labeled by mAb 3C9 (A and B) or Concanavalin A-biotin (C and D) and analyzed by flow cytometry as described in Methods section. The black lines indicate analysis of non-labeled parasites, which were identical for both lines. The figure shows one experiment representative of two independent experiments with similar results.

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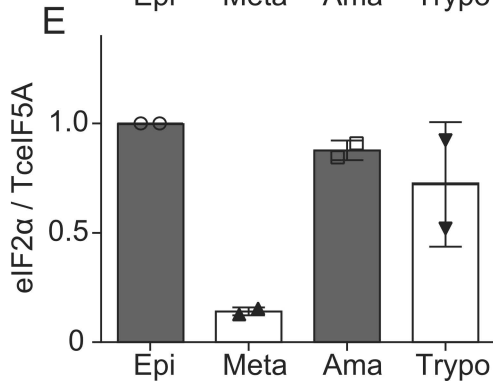
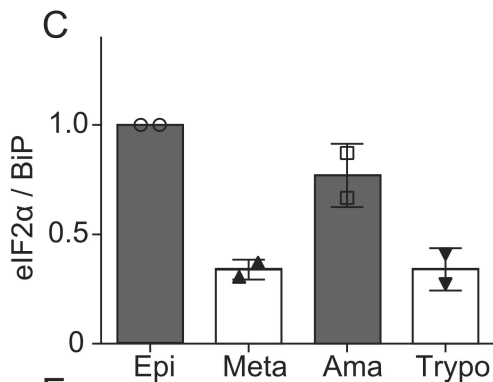
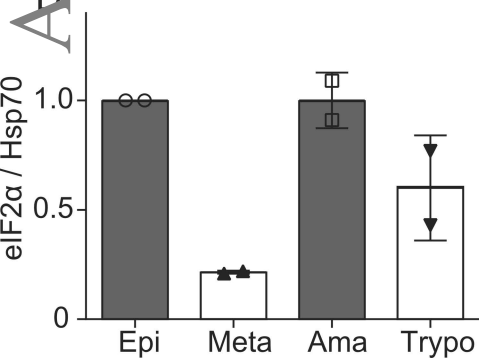
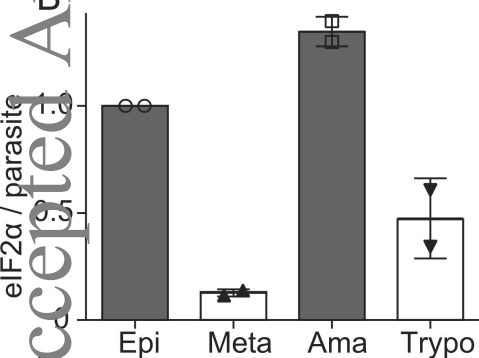
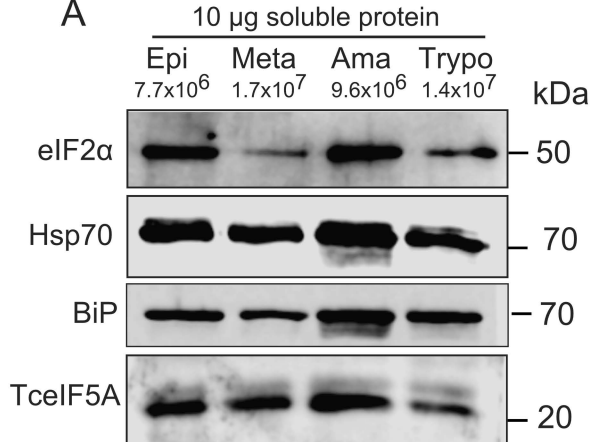
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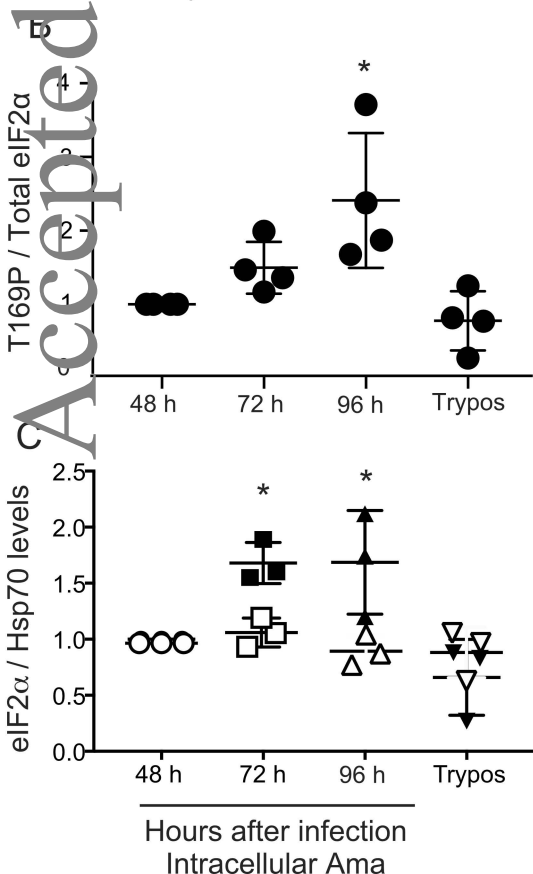
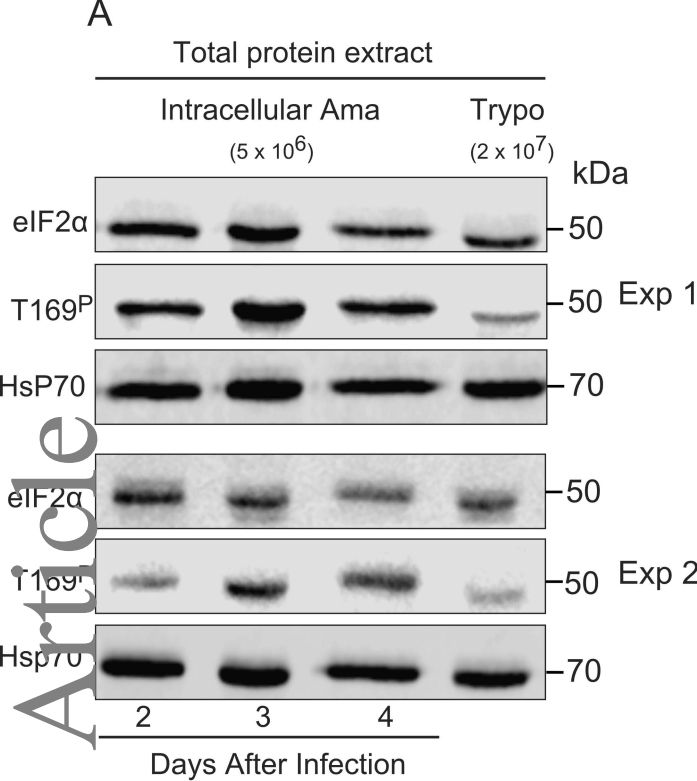
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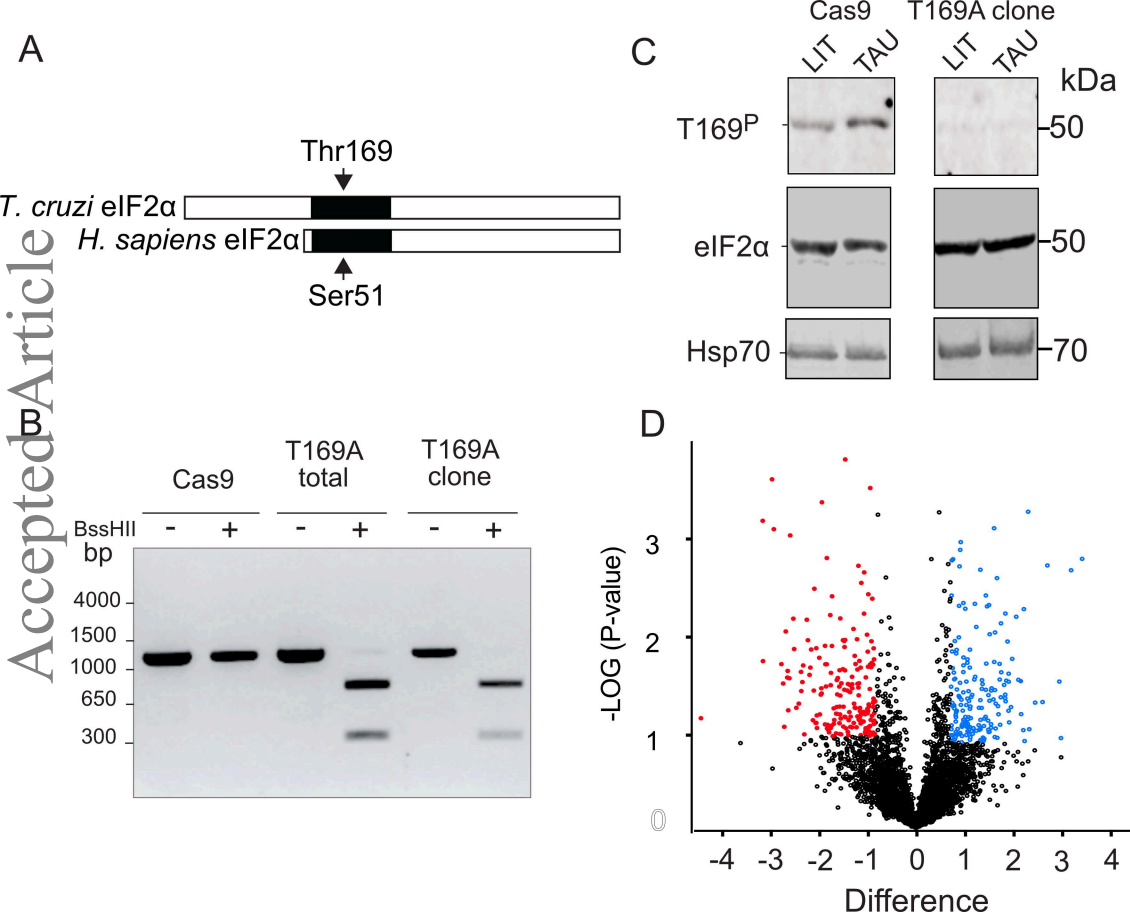
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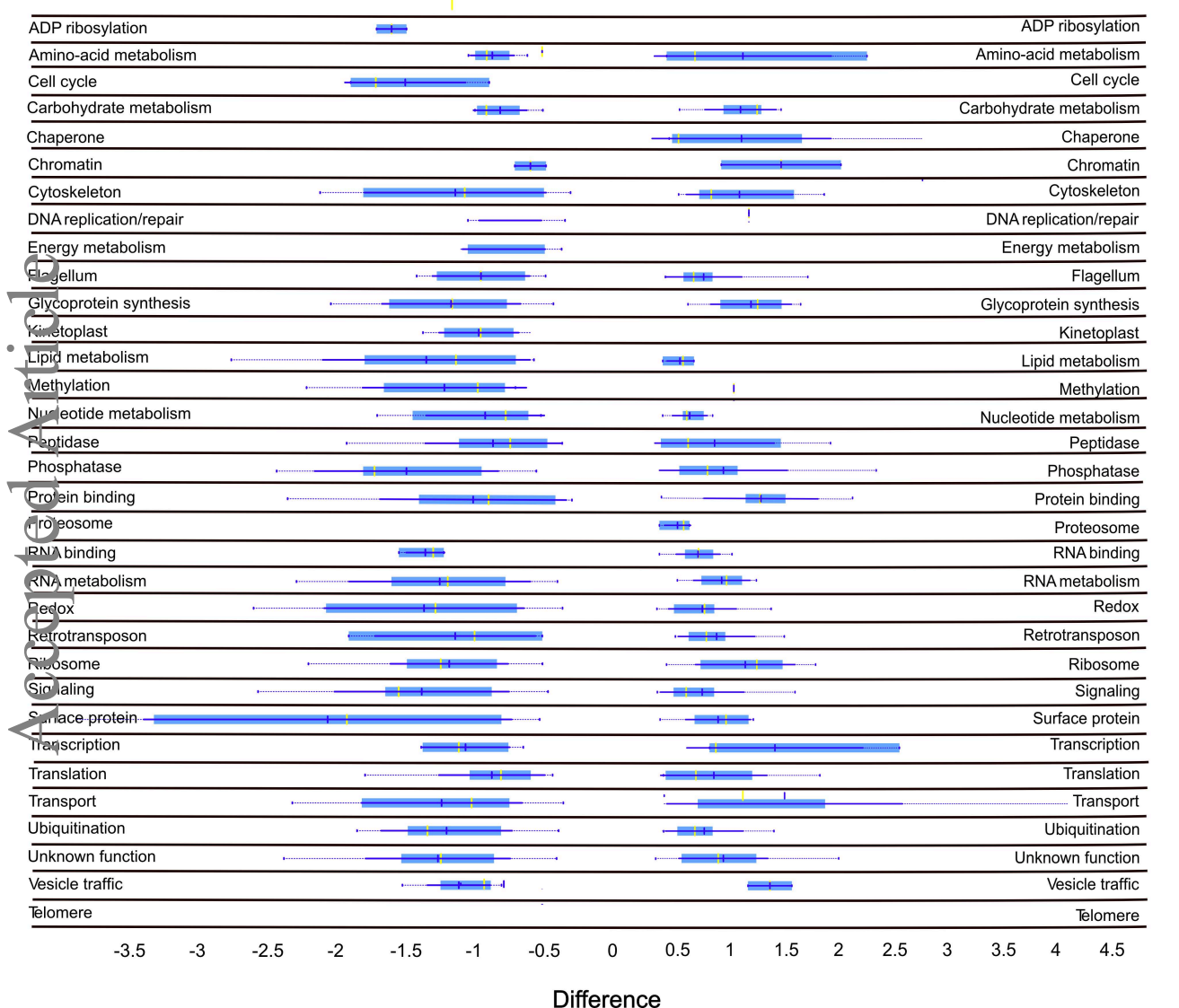
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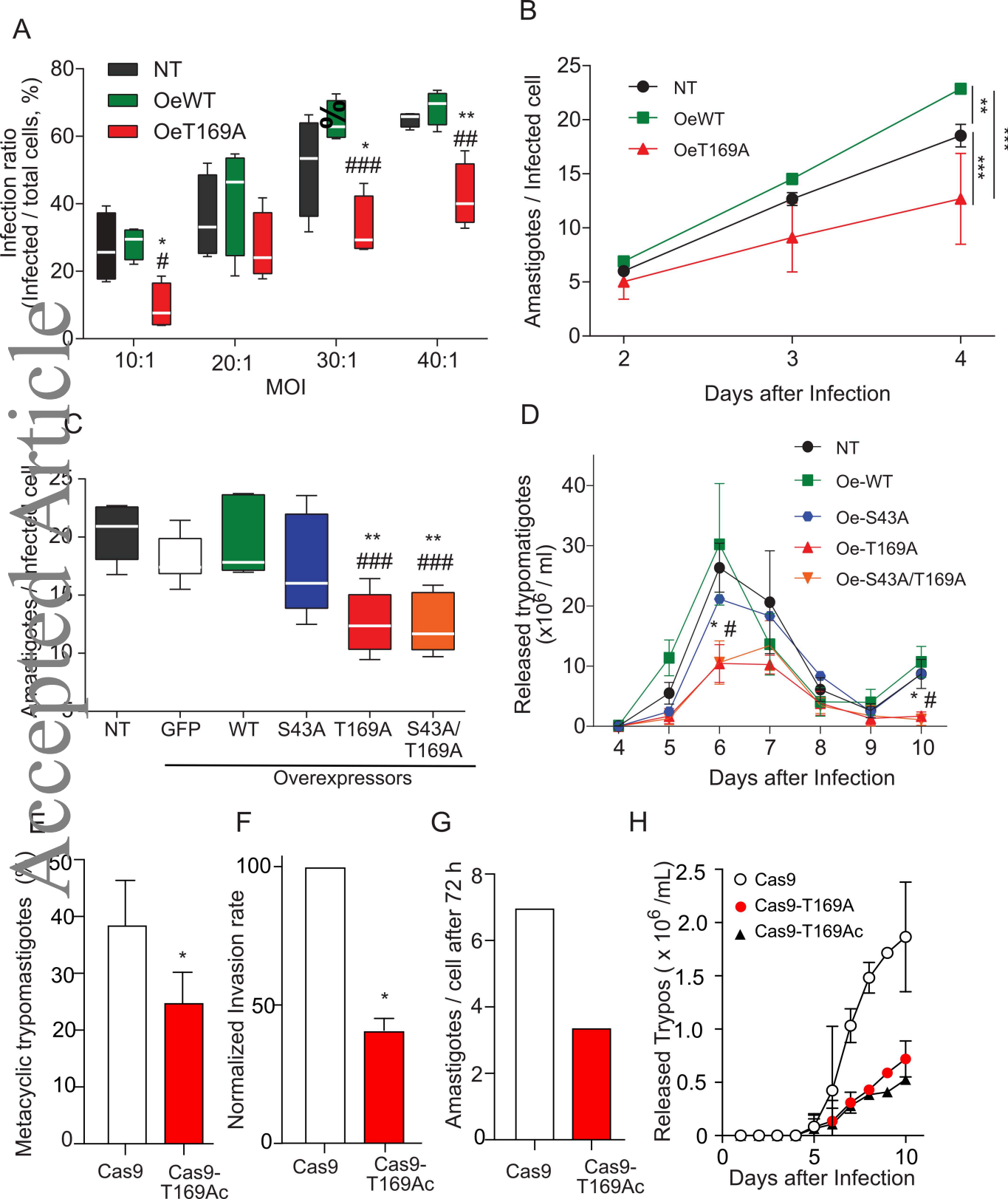
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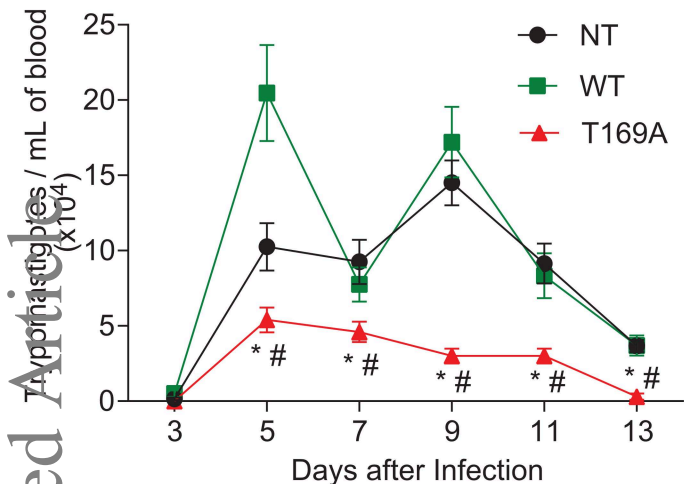




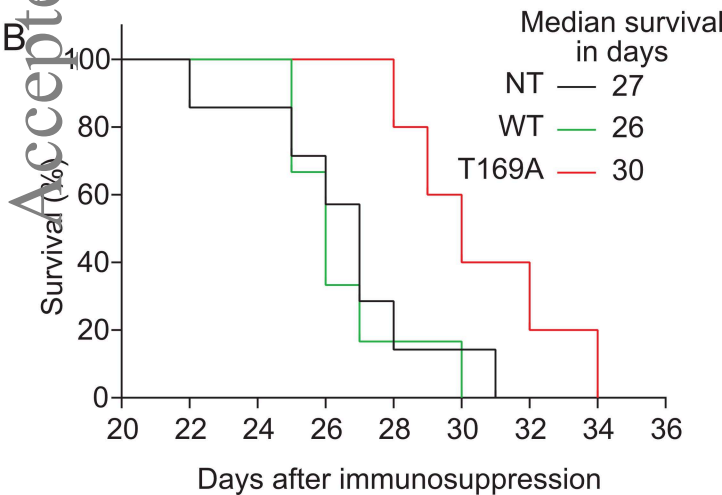




A



B



Trypsin released
6 days after
infection

Trypsin released
7 days after
infection

